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Deoxyribonucleic Acid Synthesis in FV-3-infected Mammalian Cells

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Deoxyribonucleic acid (DNA) synthesis and virus growth in frog virus 3 (FV-3)-infected mammalian cells in suspension were examined. The kinetics of thymidine incorporation into DNA was followed by fractionating infected cells. The cell fractionation procedure separated replicating viral DNA from matured virus. Incorporation of isotope into the nuclear fraction was depressed 2 to 3 hr postinfection; this inhibition did not require protein synthesis. About 3 to 4 hr postinfection, there was an increase in thymidine incorporation into both nuclear and cytoplasmic fractions. The nuclear-associating DNA had a guanine plus cytosine (GC) content of 52%; unlike host DNA it was synthesized in the presence of mitomycin C, it could be removed from nuclei by centrifugation through sucrose, and it was susceptible to nuclease digestion. This nuclear-associating DNA appeared to be a precursor of cytoplasmic DNA of infected cells. The formation of the latter DNA class could be selectively inhibited by conditions (infection at 37 C or inhibition of protein synthesis) that permit continued incorporation of thymidine into nuclear-associating DNA. The cytoplasmic DNA class also had a GC content of 52%, was resistant to nuclease degradation, and its sedimentation profile in sucrose gradients corresponded to that of infective virus. Contrary to previous reports, we found that (i) viral DNA synthesis can continue in the absence of concomitant protein synthesis, and (ii) viral DNA synthesis is not abolished at 37 C. The temperature lesion in FV-3 replication appeared to be in the packaging of DNA into the form that appears in the cytoplasmic fraction of disrupted cells.

Frog virus 3 (FV-3) is a deoxyribonucleic acid (DNA) virus that replicates in monolayer cultures of amphibian and mammalian cells maintained at 26 C (4). Virus-infected monolayer cultures are inconvenient for detailed biochemical studies on virus replication. The availability of a line of baby hamster kidney cells (BHK 21/13) that can be maintained in suspension culture afforded an opportunity to undertake such biochemical studies. As a preliminary project, an investigation of FV-3 replication and DNA synthesis in suspension cultures was undertaken.

During this investigation, we found an apparent discrepancy between the location of viral DNA synthesis as shown by autoradiography of monolayer cultures and its location as measured by incorporation of tritiated thymidine followed by fractionation of suspension cells. With the first technique, isotopically labeled DNA in infected cells was demonstrable as discrete cytoplasmic foci; with the fractionation technique, newly synthesized DNA was found to be partially located in the cytoplasm and partially associated with the nuclei of disrupted cells. Zambernard (18) has reported that FV-3 DNA synthesis occurs in the nucleus of infected fish cells. This encouraged us to consider the possibility that the locus of viral DNA synthesis or the influence of virus replication on host DNA synthesis could vary with the condition of cell culture.

The resolution of the discrepancy, which led to the demonstration of two pools of viral DNA, is described. The properties of the DNA from these pools are briefly reported, and their relevance to an understanding of FV-3 replication is discussed.

MATERIALS AND METHODS

Virus. FV-3, grown and titrated in Fathead Minnow cells (4), was used. The virus was purified before use by the following procedure. Stock virus was centrifuged once through 36% sucrose in 0.01 tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 8.5).

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The pellet was resuspended in Tris buffer, layered onto a 20 to 70% sucrose gradient, and centrifuged at 20,000 × *g* for 30 min. The virus formed a visible sharp band within the gradient; it was removed, diluted with Tris buffer (pH 7.8), and stored at −50 C.

Cells. BHK 21/13 cells, a cloned line obtained from the laboratory of Michael Stoker, were maintained as monolayers on glass. As the cells approached confluence, many were found to round up and float off the monolayer surface. Such cells (designated BHK-S) were collected and replated; stock cultures were maintained. These cells can be put into suspension and maintained in that state for at least several days. To prepare suspension cultures, the cells were removed from the monolayer with Puck's versene (Grand Island Biological Co., Grand Island, N.Y.) and were transferred to Eagle's minimal essential medium (modified for suspension cultures; Grand Island Biological Co.) containing 5% calf serum. Cells in suspension were maintained at a density of 3×10^5 to 5×10^5 per ml and were used on the first to fourth day after setting up suspension cultures. L-929 cells were maintained in suspension cultures in the medium described above.

Infection. To infect suspension cells, they were harvested and concentrated to 5×10^6 cells per ml of growth medium; then virus was added, usually at an input of 10 plaque-forming units (PFU) per cell. Adsorption was allowed to proceed for 30 min. At the end of this time, the cells were diluted to 5×10^5 per ml of growth medium.

For infection of monolayers, medium was removed, virus was added at an input of 20 PFU per cell, and adsorption was allowed to proceed for 1 hr before the medium was replaced.

Fractionation of cells. Cells were disrupted in hypotonic buffers; nuclear and cytoplasmic fractions were separated by centrifugation at 500 × *g* for 8 min (12).

Pulse-chase labeling of cells. Infected cells (8×10^7) were pulse labeled with 0.3 mc of tritiated thymidine (specific activity, 16.6 c/mmole) from 5 to 6 hr postinfection. At the end of the pulse, the cells were washed twice with medium containing 2 μg of thymidine per ml and then were resuspended in medium containing 0.5 μg of thymidine per 80 ml of medium. Samples were taken at intervals, and the cells were processed as described above.

Sucrose gradient centrifugation. Infected cells were labeled with tritiated thymidine 5 to 8 hr postinfection. Nuclear fractions or cytoplasmic fractions were layered onto 20 to 70% sucrose gradients prepared in hypotonic disruption buffer (RSB; 13) at pH 7.8. The gradients were centrifuged at 20,000 × *g* for 30 min at 10 C. Fractions (5 drops each) were collected from the bottom of each tube. Samples (0.1 ml) of each fraction were taken for determination of infectivity, and the radioactivity of the remainder was determined.

Correction in isotope assay. Radioactivity in all experiments was determined by collecting acid-precipitable material on membrane filters, then drying the filters before counting by liquid scintillation. Addition of 2 mg of tritiated *Escherichia coli* DNA (20,000 counts/min) to unlabeled nuclear and cytoplasmic fractions prior to acid precipitation and isotope assay showed that the count in the cytoplasmic fraction was exactly half that in the nuclear fraction, as a result of adsorption by other precipitable material in the cell cytoplasm. In all of the figures, counts determined for the cytoplasmic fraction should be multiplied by 2 to give a count comparable to the nuclear count.

RESULTS

Growth of FV-3 in suspension cells. BHK-S cells were harvested and infected with FV-3 at an input multiplicity of 10 PFU per cell. Nonadsorbed virus was removed by centrifugation, and the cells were resuspended in suspension medium at a final concentration of 5×10^5 cells per ml. Under these conditions, more than 95% of the cells were infected as shown by Feulgen positive cytoplasmic staining 16 hr after infection. At intervals postinfection, cells were harvested, washed with warm medium, resuspended in phosphate-buffered saline, and frozen. The next day, the frozen cells were thawed rapidly and were subjected to sonic treatment for 1 min at full power in a Raytheon sonicator. The suspension was centrifuged at 1,000 × *g* for 15 min, and samples of the supernatant fluid were assayed for infectivity. From the growth curve (Fig. 1), it is apparent that FV-3 replicated extensively in suspension cultures of BHK cells at 26 C and that most of the virus remained cell associated for the first 12 hr. About 15 hr postinfection, extensive cell disruption occurred.

Similar experiments were conducted with L-929 cells. A burst of infective virus production oc-

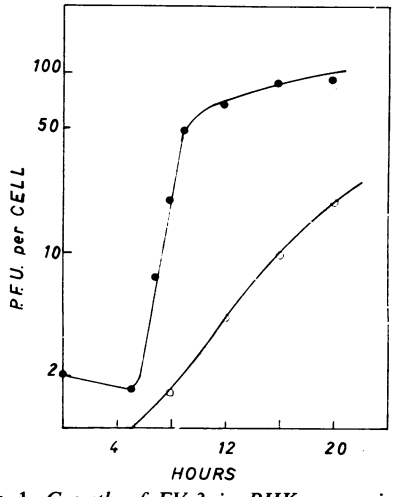

FIG. 1. *Growth of FV-3 in BHK suspension cells. Cell-associated virus* (●); *released virus* (○).

curred between 7 and 12 hr postinfection, but the yields per cell were only 6, 5, and 7 PFU per cell in three separate experiments. Adsorption studies (to be reported elsewhere) showed that virus adsorbs equally as well to L-929 cells as to BHK cells.

DNA synthesis in FV-3-infected suspension cells. Cytoplasmic DNA synthesis commenced 4 to 6 hr after FV-3 infection of BHK monolayer cells. The exact time of appearance of viral DNA apparently varied from one experiment to another; undoubtedly, this is attributable to the difficulty of simultaneously infecting all cells on a monolayer. By 5 to 6 hr postinfection, host DNA synthesis was completely inhibited (9).

Using the same technique and the same procedure with BHK-S cells in monolayer, we obtained qualitatively similar results (Fig. 2). Clearly, nuclear DNA synthesis was depressed by 3 hr postinfection. By 6 hr, nuclear DNA synthesis was completely abolished and cytoplasmic foci of thymidine-labeled DNA were obvious.

Suspension cultures of BHK or L-929 cells were infected with FV-3. At the end of the adsorption period, the cells were diluted to 5×10^5 cells per ml and tritiated thymidine (80 μc, 50 μg of thymidine per 100 ml of culture medium) was introduced. All cultures were maintained at 26 C. At intervals, 10^7 cells were removed and harvested. Cells were washed once in thymidine-saline (physiological saline containing 0.5 mg of thymidine per ml) and were disrupted as described elsewhere (13). The nuclear fraction was deposited by centrifugation (500 $\times$ *g*, 5 min) and was processed further by Penman's detergent technique (12) in order to ensure removal of contaminating cytoplasm. Nuclear and cytoplasmic fractions were adjusted to 10% trichloracetic acid, filtered, and washed on membrane filters. The filter was dried and the radioactivity was determined by liquid scintillation spectrometry.

Considering the results of autoradiographic studies with monolayer cultures and the severity of the Penman detergent technique, an unexpected finding was the incorporation of thymidine into the nuclear as well as the cytoplasmic fraction at late times (Fig. 3).

Omission of Mg^{++} from the RSB did not alter the distribution of radioactivity as was found for poxvirus-infected cells (6). Furthermore, when this procedure was tested with poxvirus-infected BHK cells, less than 10% of poxvirus DNA associated with the nuclear fraction upon disruption. The counts given in Fig. 3 are actual experimental results. For comparison with the incorporation into the nuclear fraction, the cytoplasmic counts should be multiplied by 2 to correct for self adsorbtion resulting from cytoplasmic protein (*see* Materials and Methods).

At 37 C [a temperature reported to be nonpermissive for FV-3 replication (4)], there was no incorporation of thymidine into DNA in the cytoplasmic fraction. However, in the nuclear fraction after a depression of incorporation of thymidine relative to uninfected cells, there was a marked increase in the rate of thymidine incorporation (Fig. 4) approximately 3 hr postinfection.

It was of interest to establish whether the increase in thymidine incorporation into the nuclear fraction of infected cells at either 26 or 37 C represented restimulation of host DNA synthesis or merely attachment of viral DNA to the nuclei. Autoradiography of pulse-labeled suspension cells was unsatisfactory for the following reasons: few of the infected cells attach to coverslips for subsequent fixation and autoradiography; those that do attach may not be representative of the majority of the infected population, and in any case they do not flatten out on coverslips to provide definite resolution of the site of thymidine incorporation.

To resolve the question of whether FV-3 DNA associated with the nuclei or whether host cell nuclei were continuing to synthesize DNA at late times, three experimental approaches were tried. (i) Infected BHK-S monolayer cultures (in which system only cytoplasmic DNA synthesis can be demonstrated by autoradiography at times late in infection) were fractionated by the procedures used for suspension cells. (ii) DNA synthesis in suspension cultures was inhibited by mitomycin C prior to infection and the distribution of incorporated thymidine was followed after the infection in continuing presence of mitomycin C. (iii) Removal of the newly synthesized DNA from the nuclear fraction by gentle physical methods was attempted. Procedures which provide whole nuclei free from contamination of cytoplasmic components (5) were adopted.

Fractionation of infected monolayer cultures. Confluent monolayers were infected with FV-3 and tritiated thymidine was introduced as for suspension cultures. At various times after infection, the medium was removed and the cells were rinsed in thymidine-saline. The complete monolayer culture was suspended in Puck's versene, harvested by centrifugation, disrupted in hypotonic buffer, and fractionated as described above. The results obtained were essentially the same as those described by Fig. 3A, except that the

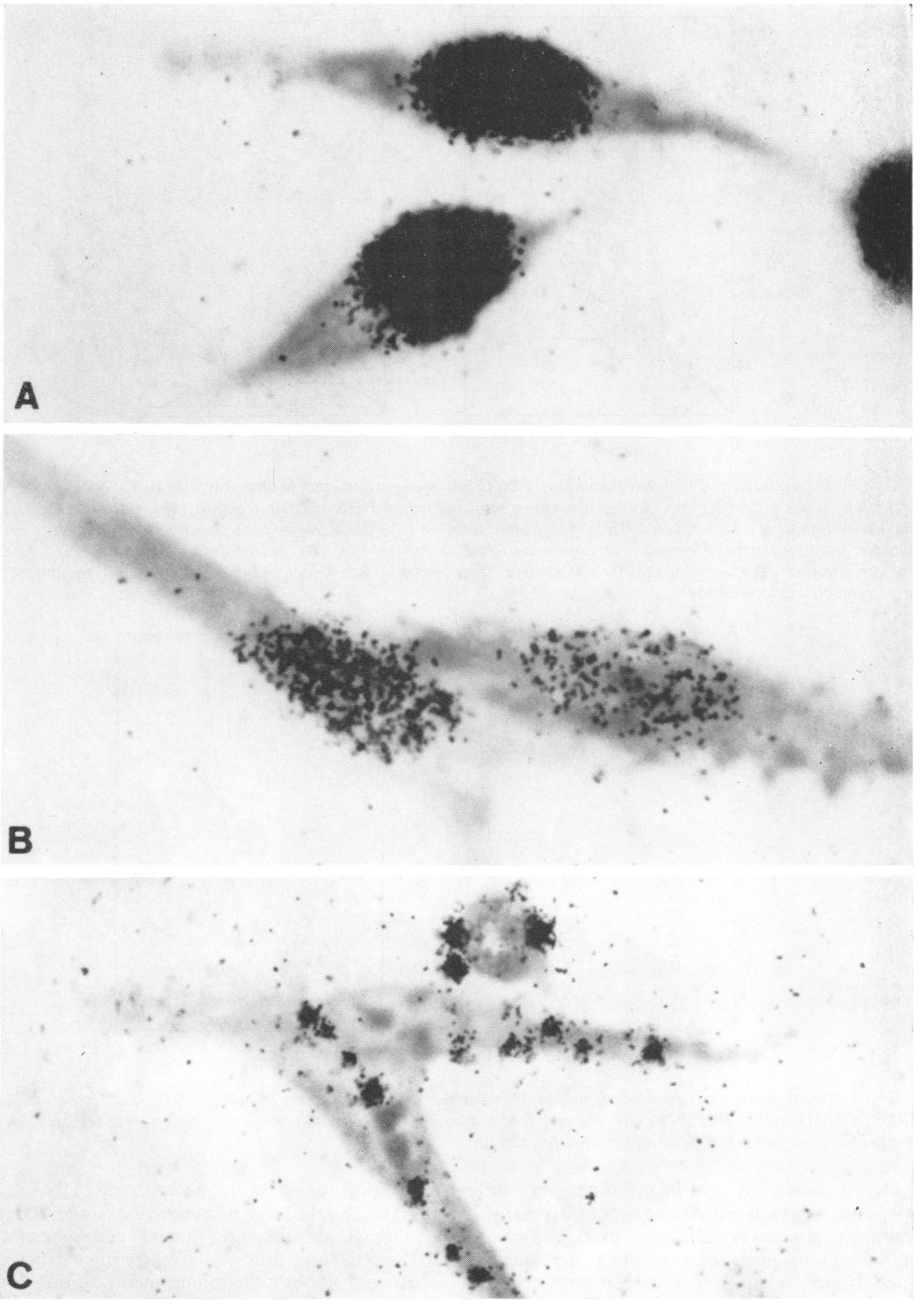

FIG. 2. *Autoradiographs of FV-3-infected BHK monolayer cultures. Cells were pulsed with tritiated thymidine (1 hr, 5 μc/ml) at various times postinfection. (A) 0 hr postinfection; (B) 3 hr postinfection; (C) 6 hr postinfection.*

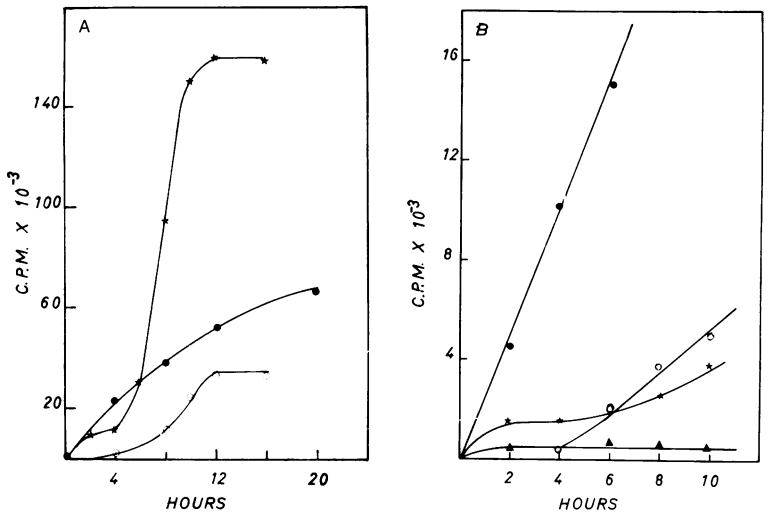

FIG. 3. *Incorporation of tritiated thymidine at 26 C into nuclear and cytoplasmic fractions of FV-3 infected cells at various times postinfection. (A) Incorporation into uninfected BHK nuclear fraction (●), into infected BHK nuclear fraction (★), into infected BHK cytoplasmic fraction (○). Base line of incorporation into uninfected cytoplasmic fraction remained constant at approximately 500 counts/min. (B) Incorporation into uninfected L-929 nuclear fraction (●), into infected L-929 nuclear fraction (★), into infected L-929 cytoplasmic fraction (○), into uninfected cytoplasmic fraction (▲).*

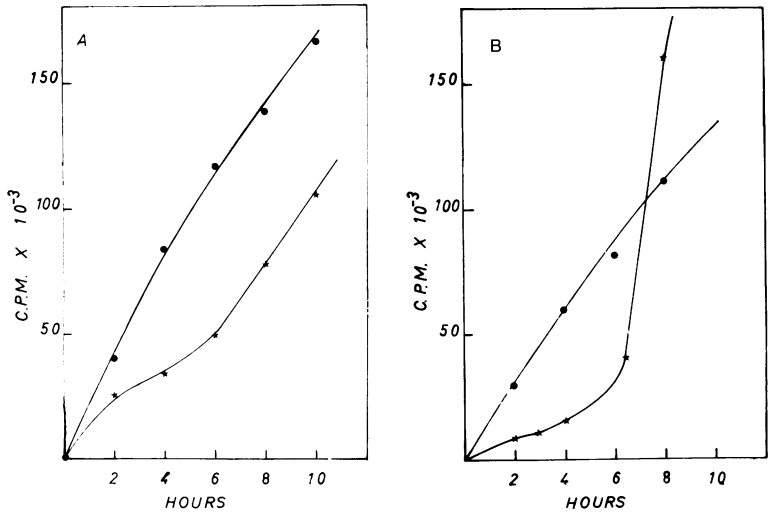

FIG. 4. *Incorporation of tritiated thymidine into nuclear and cytoplasmic fractions of FV-3-infected cells at 37 C. (A) BHK cells. (B) L-929 cells. Nuclear fraction, uninfected cells (●); nuclear fraction, infected cells (★). No increases in cytoplasmic fractions were detectable.*

nuclei of uninfected cells incorporated very little thymidine, a result which one would expect in a confluent monolayer culture. A marked increase in thymidine incorporation into the nuclear fraction was evident from 3 to 10 hr postinfection.

Effects of mitomycin C on DNA synthesis. Mitomycin C inhibits DNA synthesis in mammalian and bacterial cells but does not inhibit replication of several species viral DNA (2, 14, 15). The effect of mitomycin C on DNA synthesis in normal and virus-infected suspension BHK cells was followed to determine whether one could abolish thymidine incorporation into the nuclear fraction while permitting cytoplasmic DNA synthesis.

Suspension cells were pretreated with mito-

mycin C (10 μg/ml) for a period of 5 hr. Experiments were then conducted either with cells maintained continuously in mitomycin C or with cells from which mitomycin C was removed prior to infection. After treatment, cells were infected with FV-3 and the incorporation of thymidine into nuclear and cytoplasmic fractions was followed.

Mitomycin C depressed DNA synthesis in uninfected cells by over 95%. Synthesis of DNA in the cytoplasm commenced 3 to 4 hr after infection and proceeded just as in cells not treated with the antibiotic. A striking increase in the incorporation of thymidine into the nuclear fraction of infected cells was evident (Fig. 5). The results were similar whether mitomycin C was present continuously or was removed prior to infection.

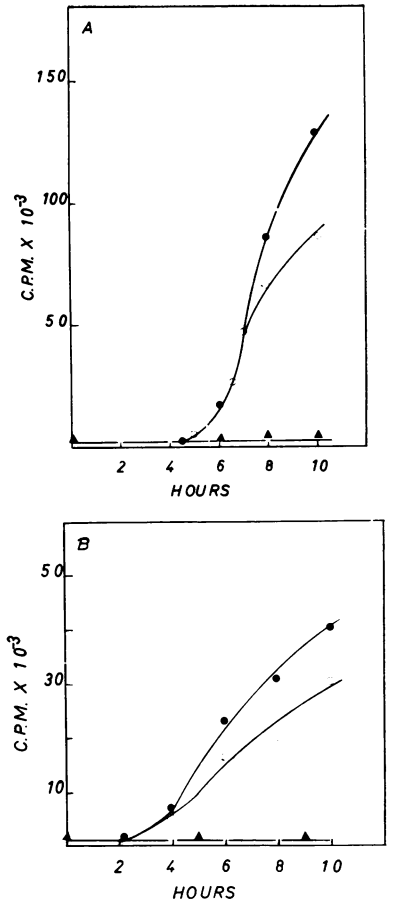

FIG. 5. *Effect of mitomycin C on thymidine incorporation into DNA in FV-3-infected BHK cells. (A) nuclear fraction. (B) Cytoplasmic fraction. Mitomycin present continuously (●); mitomycin removed at the time of infection (○); thymidine incorporation into fractions of uninfected cells (▲) with mitomycin present continuously.*

Separation of labeled DNA from nuclei. BHK-S cells in suspension (2×10^8) were infected with FV-3 (10 PFU/cell). At 5 hr postinfection, by which time synthesis of host DNA would have ceased, as judged by autoradiography of monolayer cells, tritiated thymidine (200 μc, specific activity 16.6 c/mmole) was added to the culture. Cells were harvested and fractionated 3 hr later. To remove contaminating material, nuclei were then subjected to centrifugation through sucrose according the method of Hogeboom (5). The nuclear fraction from 10^7 cells was layered over 10 ml of 0.34 M sucrose in RSB containing 0.002 M $CaCl_2$. This was then centrifuged at $600 \times g$ for 30 min. Nuclei were recovered from the pellet; over 90% of the radioactivity was recovered from the sucrose layer. Removal of label from infected nuclei was also achieved with cells infected and maintained for 10 hr at 37 C. This will be the subject of a later communication concerned with quantitating the amount of viral DNA synthesized at 37 C. In a parallel experiment, labeled nuclei from uninfected cells were quantitatively recovered and negligible loss of label to the 0.34 M sucrose layer occurred through leaching or breakage.

Comparison of nuclear-associating DNA and cytoplasmic DNA. If the nuclear-associating DNA is viral DNA, then its guanine plus cytosine (GC) content should be about 53% (11). To test for this, 2×10^8 BHK-S cells in suspension were infected with FV-3 (10 PFU/cell). Tritiated thymidine was added (100 μc, specific activity 16.6 c/mmole) 5 hr postinfection. After 3 hr, the cells were harvested and fractionated; then the nuclear-associating DNA was separated from host nuclei as described above. A portion of this DNA (100 μg) was hydrolyzed to free bases by the method of Vischer and Chargaff (16). The hydrolysate was then chromatographed by descending paper chromatography on Whatman no. 1 paper with an isopropanol-HCl-water solvent

TABLE 1. *Separation of nuclear-associating label from nuclei of infected cells*

Nuclear fraction	Total radioactivity	Radioactivity after passage through sucrose	
		Pellet	Sucrose supernatant fluid
	counts/min		
Infected cells.....	53,042	4,402	44,000
Uninfected cells...	20,253	19,560	1,120

(17). Uninfected BHK nuclear DNA was also analyzed to ensure that the method was working. The GC content of nuclear-associating DNA was found to be 52% and that of the BHK DNA 43%. These values are in agreement with previously reported values for both viral DNA and BHK DNA (11).

The two DNA pools in question were tested for their susceptibility to pancreatic deoxyribonuclease degradation. A suspension culture of 2×10^8 BHK cells was infected with FV-3. At 6 hr postinfection, 100 μc of tritiated thymidine (specific activity, 6.7 c/mmole) was introduced. Two hours later, the cells were harvested, washed with thymidine-saline, and fractionated into nuclear and cytoplasmic fractions. Nuclear-associated DNA was separated from nuclei as described above, and samples of the sucrose supernatant fluid or of the cytoplasmic fraction were incubated at 37 C with pancreatic deoxyribonuclease (100 μg/ml) plus $MgCl_2$ at a final concentration of 10^{-3} M. Samples of the reaction mixture were withdrawn at intervals and the decrease in acid-precipitable radioactivity was determined. The results (Table 2) showed that the cytoplasmic class was almost completely resistant to degradation, whereas the nuclear species was completely degradable.

The sedimentation patterns of nuclear-associated DNA and cytoplasmic DNA in sucrose gradients were compared (Fig. 6). The results indicated that the cytoplasmic DNA fraction sedimented much faster than the nuclear-associating species and was coincident with infectivity. We conclude that the DNA of the cytoplasmic fraction is in the form of virus particles or at least in a form that sediments with the same characteristics as virus particles.

Precursor product relation between nuclear-associated and cytoplasmic DNA. If the nuclear-associating DNA fraction represents viral DNA that is eventually packaged to yield a DNA pool with the characteristics of DNA in the cytoplasmic fraction, then it should be possible to demonstrate movement of isotopic thymidine from one pool to the other. To examine this, a pulse-chase experiment was conducted. The results (Fig. 7) indicated that the loss of label from the nuclear fraction can be correlated with a rise in label in the cytoplasmic fraction. Cor-

TABLE 2. *Degradation of DNA fractions by exogenous nuclease*

Time of incubation (min)	Acid-precipitable radioactivity (% of maximum)	
	Cytoplasmic fraction	Nuclear fraction
0	100	100
15	96	50
30	93	20
60	93	15

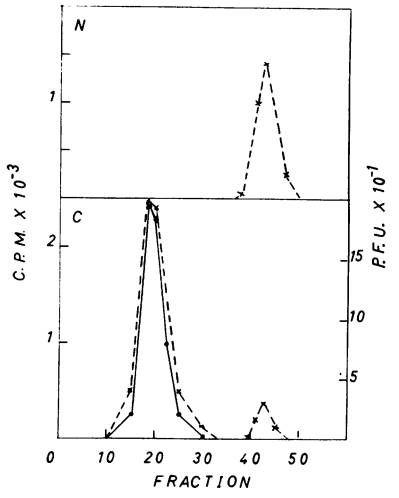

FIG. 6. *Sedimentation of tritiated thymidine-labeled material from nuclear or cytoplasmic fractions of infected BHK cells. Upper portion of graph (N) depicts nuclear associated material; lower portion (C) depicts the cytoplasmic material. Broken lines represent counts/min and solid line represents PFU.*

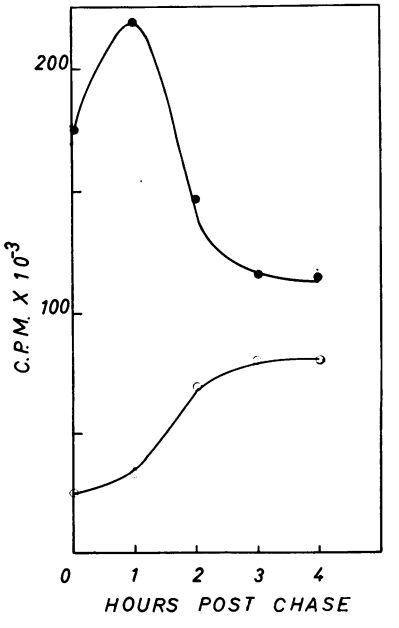

FIG. 7. *Precursor-product relation between nuclear-associated and cytoplasmic DNA. Pulse-chase experiment was described in Materials and Methods. Abscissa represents time after transfer to unlabeled thymidine. Change in radioactivity in nuclear fraction (●) or cytoplasmic fraction (○).*

recting for self-adsorption in the assay of the cytoplasmic fraction (factor of 2), the rise in cytoplasmic count was equal to the loss in count observed in the nuclear fraction.

Effect of inhibitors of protein synthesis on DNA synthesis. Streptovitacin (a hydroxylated cycloheximide, The Upjohn Co., Kalamazoo, Mich.) is a potent inhibitor of protein synthesis. At concentrations of 50 μg/ml, protein synthesis in infected BHK-S cells, as measured by C^{14}-leucine uptake, was abolished within 1 hr. Normal and FV-3-infected cultures were established, and tritiated thymidine was introduced as described above. At 0, 1.5, or 6 hr postinfection, streptovitacin (50 μg/ml) was added to the cultures. At intervals, the incorporation of label into nuclear and cytoplasmic fractions was followed. The results of a typical experiment are given in Fig. 8. The main point we wish to make is that when streptovitacin was added 6 hr postinfection, incorporation of label into the cytoplasmic fraction was rapidly arrested, but incorporation of label into the nuclear fraction continued. When label was added prior to the detectable onset of viral DNA synthesis, there was negligible incorporation of label into either fraction. Incorporation of label into nuclei of uninfected cells was not markedly affected by streptovitacin for at least 4 hr after its introduction. Another point of interest is that depression of uptake of label into the nuclear fraction of infected cells is even more marked when protein synthesis is blocked at the time of infection.

Interrelationship of protein synthesis and DNA synthesis. We investigated the question of whether protein(s) necessary for DNA replication can accumulate in the absence of DNA synthesis. The procedure used was essentially that described by Kates and McAuslan (8); DNA synthesis was blocked and after 5.5 hr the inhibitor of DNA synthesis [in this case hydroxyurea, 25 μg/ml, as used by Kucera and Granoff (9)] was removed. At the same time, an inhibitor of protein synthesis (streptovitacin, 50 μg/ml) was introduced and the incorporation of tritiated thymidine into acid-insoluble material was followed. Our results (Fig. 9) indicated that the appearance of label in the cytoplasmic fraction (mature virus) is blocked as expected, but incorporation of label into the nuclear-associating DNA (replicating DNA) occurs and continues for several hours.

DISCUSSION

We found suspension cultures of BHK 21/13 cells convenient for studying the details of FV-3 DNA replication. Suspension cells could be infected to provide single-step growth conditions and the sequence of events could be sharply and reproducibly defined. The yield of infective virus was approximately 150 to 200 PFU per cell by 12 to 15 hr postinfection. A low level of virus production and DNA synthesis at 26 C was detected in L-929 cells. The main features of FV-3 DNA replication in BHK cells, based on the preceding results, are as follows.

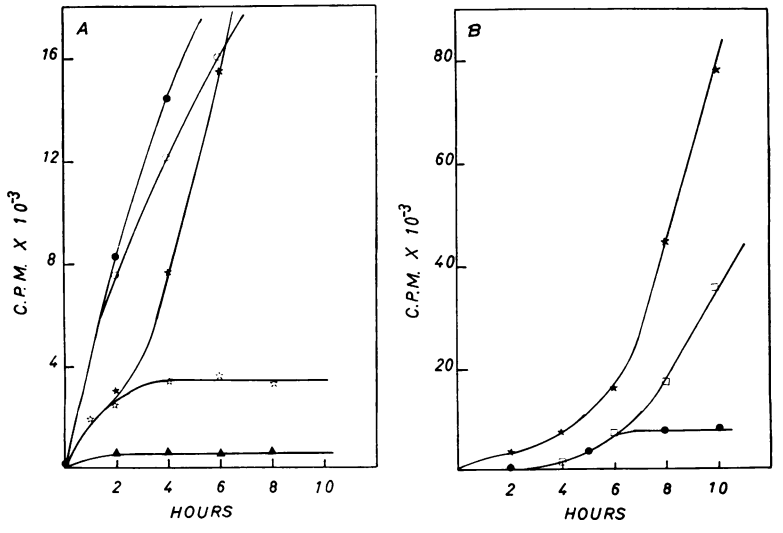

FIG. 8. *Effect of inhibition of protein synthesis at various times postinfection on incorporation of tritiated thymidine. (A) Nuclear fraction of uninfected cell in the absence of streptovitacin (●) or with streptovitacin added at 1 hr postinfection (○). Nuclear fraction of FV-3-infected BHK cells with streptovitacin added at time 0 (▲) at 1.5 hr postinfection (☆), or at 6 hr postinfection (★). (B) Nuclear fraction of infected cells with streptovitacin added at 6 hr postinfection or in the absence of streptovitacin (★). Cytoplasmic fraction of infected cells in the absence of streptovitacin (□) or with streptovitacin added at 6 hr postinfection (●).*

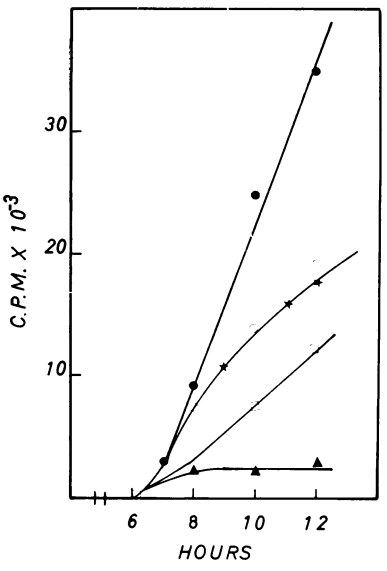

FIG. 9. *Capacity to initiate FV-3 DNA synthesis after release of hydroxyurea inhibition in the presence of an inhibitor of protein synthesis. Tritiated thymidine was added at 6 hr postinfection. Nuclear fraction, hydroxyurea was added at 0 to 5.5 hr postinfection, then removed (●); nuclear fraction, hydroxyurea added to 0 to 5.5 hr postinfection, streptovitacin (50 μg/ml) added at 5.5 to 12 hr postinfection (○); nuclear fraction no hydroxyurea but streptovitacin added at 5.5 to 12 hr postinfection (★); cytoplasmic fraction, hydroxyurea added 0 to 5.5 hr postinfection (□); cytoplasmic fraction, hydroxyurea added 0 to 5.5 hr postinfection, streptovitacin added at 5.5 to 12 hr postinfection (▲).*

Host DNA synthesis is inhibited by infection within the first 2 to 3 hr. In BHK cells, inhibition tends to be obscured by the increase in nuclear-associated DNA, but inhibition is marked in the L-929 system. This inhibition does not require protein synthesis; in fact, inhibition is potentiated in the absence of protein synthesis (Fig. 8). Thus, this system differs from pseudorabies (1) or poxvirus (10) in its capacity to block host DNA synthesis in the absence of protein synthesis.

Two pools of viral DNA occur in infected cells. One of these associates with nuclei upon disruption of cells in hypotonic buffer, the other remains with the cytoplasmic fraction. The nuclear-associating DNA is susceptible to nuclease degradation (Table 2), is synthesized in the presence of mitomycin C, and can be readily separated from nuclei. From its GC content (52%) and from precursor product studies, we believe that it is viral DNA [53% GC (11)] rather than host DNA [43% (11)]. The cytoplasmic DNA is not susceptible to nuclease and sediments in sucrose with infectivity (Fig. 6). During virus maturation, it appears that replicating viral DNA is in excess and is not completely withdrawn for packaging into virus. It is noteworthy that the superb electron micrographs taken by Darlington (3) show areas in the cytoplasm surrounded by mitochondria. These areas were referred to as possible synthesis (s) sites rather than as the loci where packaging and arrangement of crystalline particles were observed. We tentatively suggest that the (s) areas are the sites of DNA replication and that these areas become associated with nuclei upon disruption of cells either because of the affinity of native viral DNA for the nuclear membrane or because the organelles surrounding these sites attach to nuclei.

The onset of viral DNA replication requires protein synthesis. Once viral DNA synthesis is initiated it can proceed for several hours in the absence of concomitant protein synthesis (Fig. 8). This finding contradicts the results of Kucera and Granoff (9). The proteins necessary for DNA synthesis can accumulate under the direction of input DNA templates; thus, if inhibitors of DNA synthesis are removed but protein synthesis is then blocked, DNA synthesis can initiate and proceed for several hours (Fig. 9). Again, this is in contradiction to the findings of Kucera and Granoff (9). It should be noted that in the experiments illustrated in Fig. 9, DNA synthesis was inhibited with hydroxyurea for only part (about one-fifth) of the normal DNA synthetic period so that there was only a limited period in which proteins for DNA synthesis could accumulate. On the other hand, in the original experiments with poxvirus (8), proteins necessary for DNA synthesis were allowed to accumulate over the entire normal DNA replication period before proceeding with the experiment.

The maturation of viral DNA into particles (i.e., the appearance of cytoplasmic DNA) is highly sensitive to inhibitors of protein synthesis (Fig. 8). Maturation can be rapidly blocked by levels of streptovitacin (10 μg/ml) that do not markedly depress net protein synthesis for several hours. Therefore, at least one structural viral protein is limiting and is not made in excess by replicating DNA. At nonpermissive temperatures (37 C), viral DNA is synthesized (Fig. 4), as shown by the synthesis of nuclear-associating DNA, but it is not packaged into a form that appears in the cytoplasmic fraction. The amount of viral DNA synthesized in BHK cells at 37 C may be much smaller than the

amount synthesized at 26 C. This may or may not be true for L-929 cells, in which the incorporation of label into the nuclear-associating DNA is considerably higher at 37 C than it is at 26 C. Application of the technique of Jungwirth and Dawid (7) should provide a convenient method to resolve this question.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Ben-Porat, T., and A. S. Kaplan. 1965. Mechanism of inhibition of cellular DNA synthesis by pseudorabies-virus. Virology **25**:22–29.
2. Ben-Porat, T., M. Reissig, and A. S. Kaplan. 1961. Effect of mitomycin C on the synthesis of infective virus and deoxyribonucleic acid in pseudorabies virus-infected rabbit kidney cells. Nature **190**:33–34.
3. Darlington, R. W., A. Granoff, and D. C. Breese. 1966. Viruses and renal carcinoma of *Rana pipiens*. II. Ultrastructural studies and sequential development of virus isolated from normal and tumor tissue. Virology **29**:149–156.
4. Granoff, A., P. Came, and D. Breese. 1966. Viruses and renal carcinoma of *Rana pipiens*. I. The isolation and properties of virus from normal and tumor tissue. Virology **29**:133–148.
5. Hogeboom, G. H., W. C. Schneider, and M. J. Striebich. 1952. Cytochemical studies. V. On the isolation and biochemical properties of liver cell nuclei. J. Biol. Chem. **196**:111–120.
6. Joklik, W. K., and Y. Becker. 1964. The replication and coating of vaccinia DNA. J. Mol. Biol. **10**:452–474.
7. Jungwirth, C., and I. B. Dawid. 1967. Vaccinia DNA: Separation of viral from host cell DNA. Arch. Ges. Virusforsch. **20**:464–468.
8. Kates, J. R., and B. R. McAuslan. 1967. Relationship between protein synthesis and viral deoxyribonucleic acid synthesis. J. Virol. **1**:110–114.
9. Kucera, L., and A. Granoff. 1968. Viruses and renal carcinoma of *Rana pipiens*. VI. Interrelationships of macromolecular synthesis and infectious virus production in frog virus 3-infected BHK 21/13 cells. Virology **34**:240–249.
10. Loh, P. C., and F. E. Payne. 1965. Effect of *p*-fluorophenylalanine on the synthesis of vaccinia virus. Virology **25**:560–574.
11. Maes, R., and A. Granoff. 1967. Viruses and renal carcinoma of *Rana pipiens*. IV. Nucleic acid synthesis in frog virus 3-infected BHK cells. Virology **33**:491–502.
12. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. **17**:117–130.
13. Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger-RNA. Proc. Natl. Acad. Sci. U.S. **49**:654–662.
14. Sekiguchi, M., and Y. Takagi. 1960. The effect of mitomycin C on the synthesis of bacterial and viral deoxyribonucleic acid. Biochim. Biophys. Acta **41**:434–443.
15. Shiba, S., A. Terawaki, T. Tayuchi, and J. Kawamata. 1959. Selective inhibition of formation of deoxyribonucleic acid in *Escherichia coli* by mitomycin C. Nature **183**:1056–1057.
16. Vischer, E., and E. Chargaff. 1948. The composition of pentose nucleic acids of yeast and pancreas. J. Biol. Chem. **176**:715–734.
17. Wyatt, G. 1951. The purine and pyrimidine composition of deoxypentose nucleic acids. Biochem. J. **48**:584–590.
18. Zambernard, J. 1967. The effect of *p*-fluorophenylalanine and hydroxyurea on the replication of a frog kidney virus. J. Cell Biol. **35**: 191A–192A.